Mother liquors are defined as the solutions that contain all compounds (buffer, crystallizing agent, *etc.*) at the final concentration for crystallization except the macromolecule. Samples of macromolecules often contain quantities of salt of unknown composition, and it is therefore wise to dialyse new batches against well characterized buffers. Whatever the crystallization method used, it almost always requires a high concentration of macromolecule. This may imply concentration steps using devices operating under nitrogen pressure, by centrifugation, or by lyophilization (notice that lyophylization may denature proteins and that non-volatile salts also lyophilize and will accumulate). Dialysis against high-molecular-weight PEG may also be used. During concentration, pH and ionic strength may vary and, if not kept at the appropriate values, denaturation of samples may occur.

4.1.4.4. *Strategic concerns: a summary*

Homogeneity: Perhaps the most important property of a system to be crystallized is its purity. Crystallization presupposes that identical units are available for incorporation into a periodic lattice. If crystallization fails, reconsidering purification protocols often helps achieve success.

Stability: No homogeneous molecular population can remain so if its members alter their form, folding, or association state. Hence, it is crucial that macromolecules in solution are not allowed to denature, aggregate, or undergo conformational changes.

Solubility: Before a molecule can be crystallized, it must be solubilized. This means creation of monodisperse solutions free from aggregates and molecular clusters. Solubility and crystallizability strongly depend on substances (organic solvents and PEGs) that reduce the ionic strength of the solution (Papanikolau & Kokkinidis, 1997).

Supersaturation: Crystals grow from systems displaced from equilibrium so that restoration requires formation of the solid state. Thus, the first task is to find ways to alter the properties of the crystallizing solutions, such as by pH or temperature change, to create supersaturated states.

Association: In forming crystals, molecules organize themselves through self-association to produce periodically repeating three-dimensional arrays. Thus, it is necessary to facilitate positive molecular interactions while avoiding the formation of precipitate or unspecific aggregates, or phase separation.

Nucleation: The number, size and quality of crystals depend on the mechanisms and rates of nuclei formation. In crystallization for diffraction work, one must seek to induce limited nucleation by adjustment of the physical and chemical properties of the system.

Variety: Macromolecules may crystallize under a wide spectrum of conditions and form many polymorphs. Thus, one should explore as many opportunities for crystallization as possible and explore the widest spectrum of biochemical, chemical and physical parameters.

Control: The ultimate value of any crystal is dependent on its perfection. Perturbations of the mother liquor are, in general, deleterious. Thus, crystallizing systems have to be maintained at an optimal state, without fluctuations or shock, until the crystals have matured.

Impurities: Impurities can contribute to a failure to nucleate or grow quality crystals. Thus, one must discourage their presence in the mother liquor and their incorporation into the lattice.

Perfection: Crystallization conditions should be such as to favour crystal perfection, to minimize defects and high mosaicity of the growing crystals, and to minimize internal stress and the incorporation of impurities. Predictions from crystal-growth theories may help to define such conditions (Chernov, 1997*b*, 1999).

Preservation: Macromolecular crystals may degrade and lose diffraction quality upon ageing. Thus, once grown, crystals may be stabilized by temperature change, addition of more crystallizing agent, or by some other suitable alteration in the mother liquor.

4.1.5. Techniques for physical characterization of crystallization

Crystallization comprises four stages. These are prenucleation, nucleation, growth and cessation of growth. It proceeds from macromolecules in a solution phase that then 'aggregate' upon entering a supersaturated state and which eventually undergo a phase transition. This leads to nuclei formation and ultimately to crystals that grow by different mechanisms. Each of these stages can be monitored by specific physical techniques. Although systematic characterization of crystallization is usually not carried out in practice, characterization of individual steps and measurement of the physical properties of crystals obtained under various conditions may help in the design of appropriate experimental conditions to obtain crystals of a desired quality (*e.g.* of larger size, improved morphology, increased resolution or greater perfection) reproducibly.

4.1.5.1. *Techniques for studying prenucleation and nucleation*

Dynamic light scattering (DLS) relies on the scattering of monochromatic light by aggregates or particles moving in solution. Because the diffusivity of the particles is a function of their size, measurement of diffusion coefficients can be translated into hydrodynamic radii using the Stokes–Einstein equation. By making measurements as a function of scattering angle, information regarding aggregate shape can also be obtained. For single-component systems, the method is straightforward for determining the size of macromolecules, viruses and larger particles up to a few μm. For polydisperse and concentrated systems, the problem is more complex, but with the use of autocorrelation functions and advances in signal detection (Peters *et al.*, 1998), DLS provides good estimates of aggregate-size distribution.

In bio-crystallogenesis, investigations based on light scattering have been informative in delineating events prior to the appearance of crystals subsequently observable under the light microscope, that is, the understanding of prenucleation and nucleation processes. Many studies have been carried out with lysozyme as the model (Kam *et al.*, 1978; Durbin & Feher, 1996), though not exclusively, and they have developed with two objectives. One is to analyse the kinetics and the distribution of molecular-aggregate sizes as a function of supersaturation. The aim is to understand the nature of the prenuclear clusters that form in solution and how they transform into crystal nuclei (Kam *et al.*, 1978; Georgalis *et al.*, 1993; Malkin & McPherson, 1993, 1994; Malkin *et al.*, 1993). Such a quantitative approach has sought to define the underlying kinetic and thermodynamic parameters that govern the nucleation process. The second objective is to use light-scattering methods to predict which combinations of precipitants, additives and physical parameters are most likely to lead to the nucleation and growth of crystals (Baldwin *et al.*, 1986; Mikol, Hirsch & Giegé, 1990; Thibault *et al.*, 1992; Ferré D'Amaré & Burley, 1997). A major goal here is to reduce the number of empirical trials. The analyses depend on the likelihood that precipitates are usually linear, branched and extended in shape, since they represent a kind of random polymerization process (Kam *et al.*, 1978). Aggregates leading to nuclei, on the other hand, tend to be more globular and three-dimensional in form. Thus, a mother liquor that indicates a nascent precipitate can be identified as a failure, while those that have the character of globular aggregates hold promise for further exploration and refinement. Other analyses have been based on discrimination between polydisperse and monodisperse protein

references